ENZYMATIC DECOMPOSITION OF NATIVE PROTEIN

By LILA MILLER

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ENZYMATIC HYDROLYSIS OF LACTALBUMIN*

BY LILA MILLER AND HERBERT O. CALVERY

(From the Department of Biological Chemistry, Medical School, University of Michigan, Ann Arbor)

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Lactalbumin was first separated from whey and identified as an individual protein in 1885 (1). Since then the chief studies with this protein have been concerned with its nutritive value and its relationship to serum albumin, since the latter was thought to be a possible precursor of lactalbumin. Outside of these two phases, lactalbumin has been studied relatively little, and no extensive enzymatic studies have been reported. The most recent one was a comparative study on lactalbumin and casein with unfractionated extracts of the pancreas and intestinal mucosa (2).

With the development of the adsorption methods for the separation of enzymes, it is now possible to study the enzymatic degradation of proteins in successive steps. By such a procedure a fairly complete picture of the structure of some of the protamines has been obtained (3, 4). The structure of the more complex proteins may be partially elucidated by similar studies which would involve an investigation of the successive actions of the individual enzymes and the products in each instance. Only one protein of high molecular weight, ovalbumin, has been so studied (5), and in this case, the second phase has not been completed.

The purpose of this investigation was to subject lactalbumin to the successive actions of pepsin, the enzymes of the pancreas, and finally those of the intestinal mucosa, and thus to ascertain the type of linkage opened and the extent of hydrolysis with each enzyme preparation used. In this manner the foundation

* A preliminary report was presented before the American Society of Biological Chemists at Detroit, April 10–13, 1935 (*Proc. Am. Soc. Biol. Chem.*, **8**, lxvi (1935); *J. Biol. Chem.*, **109** (1935)).

for a more detailed study of a second protein of high molecular weight was laid.

EXPERIMENTAL

Materials—The lactalbumin[1] contained 14.84 per cent nitrogen and 0.66 per cent ash, and at least 7.11 per cent of the nitrogen was in the form of free amino groups. These values and all others reported in this paper have been corrected for moisture and ash. The protein was passed through an 80 mesh screen and then pulverized in a ball mill to an impalpable powder. Suspensions of this material could then be sampled satisfactorily.

A commercial preparation of pepsin (Difco, 1:20,000) was used rather than the crystalline enzyme, since the preparation of crystalline pepsin involves so great a loss of material, which is of significance where large quantities are required. Also, the crystalline and the commercial pepsin have been found to hydrolyze ovalbumin to the same extent (6), and it seemed reasonable to assume that the same would hold in the case of lactalbumin. Incidentally, the studies with which this one is to be compared were not made with crystalline pepsin but with a commercial product.

The enzymes of the pancreas and intestinal mucosa of pigs were separated by selective adsorption methods. The extracts were prepared in the customary manner, as described by Waldschmidt-Leitz (7). The methods of separation and determination of the activity of the various enzymes were those described by Calvery (5). One change was made in the procedure for the test for aminopolypeptidase and dipeptidase; namely, that only one-tenth of the quantity of the substrate and enzyme and 0.02 N solutions of base were used. In one preparation of protaminase, the inactive trypsin was removed by adsorbing it on egg albumin and then precipitating the latter with acetone (8).

Methods

The extent of hydrolysis in all instances was estimated by the Van Slyke amino nitrogen determination. The micromethod was carried out in the customary manner, with a 3 minute reaction period. This was considered adequate for a complete reaction,

[1] The lactalbumin was a commercial preparation obtained from the Harris Laboratories, Tuckahoe, New York.

as the room temperature ranged from 26–28° and the same results were obtained in the 3 and the 30 minute periods with undigested lactalbumin.

For the comparison of the carboxyl and amino groups liberated, the titrimetric methods of Henriques and Sørensen (9), Willstätter and Waldschmidt-Leitz (10), and the "modified Harris," as well as the Van Slyke gasometric method were used. These titrations were carried out on a semimicro scale; *i.e.*, 5 cc. of the hydrolysate which contained approximately 27 mg. of substrate nitrogen were titrated with 0.02 or 0.03 N solutions. To stop the hydrolysis, the aliquots for titration were mixed with 10 or 20 cc. of 0.149 N alkali in the case of the Sørensen method and with 50 cc. of 95 per cent alcohol in the other titrations.

The titration described by Harris consists of two stages (11). In the first, the alcoholic solution is titrated with 0.1 N aqueous sodium hydroxide to the end-point of thymolphthalein; in the second, this mixture is titrated with a similar solution of hydrochloric acid from the alkaline pH to the end-point of methyl red. The titration designated as "modified Harris" refers to the second stage. The modification is the substitution of an 0.02 N alcoholic solution of hydrochloric acid for a 0.1 N aqueous solution and the replacement of the first stage by the Willstätter method. Thus, at the end of the Willstätter titration methyl red or methyl red-methylene blue was added to the solution and the titration carried to the end-point by the addition of the alcoholic acid solution.

For the Willstätter and modified Harris titration, the standard used for comparison of the end-point consisted of 5 cc. of an aqueous 0.02 N solution of pure alanine, 50 cc. of 95 per cent alcohol, the proper indicators, and a quantity of alkali or acid equal to the theoretical plus the blank. This standard was found to be satisfactory with respect to color, as it did not fade for several hours provided the solution was kept in a closed vessel. All titration data have been corrected for the blank obtained by the substitution of 5 cc. of water for the hydrolysate. All the titration methods gave theoretical results with pure alanine.

General Procedures

All the digestions were carried out at 30° in a thermostat. If observations on the early stages of hydrolysis were to be made,

the enzyme and substrate solutions and suspensions were brought to this temperature before they were mixed. Toluene was used as a preservative during all experiments and storage of the hydrolysates.

An enzyme control was carried out with every hydrolysis. In the case of peptic hydrolysis, the control consisted of pepsin and acid in the same concentrations as those mixed with the substrate. In the subsequent experiments, these autolysates of pepsin were treated with enzymes in the same manner as the peptic hydrolysates of lactalbumin.

The data obtained by the Van Slyke method and the titrations represent averages of duplicate or triplicate determinations, except in a few cases in which the material was insufficient for two titrations and in the early stages of peptic hydrolysis where the changes were very rapid. All values have been corrected for the blanks. In the check determinations in the Van Slyke method, the limit of differences allowed was 0.02 cc. of gas, most values being in a closer agreement than this arbitrary limit.

Throughout this paper the extent of hydrolysis, *i.e.* the increase in amino nitrogen as determined by the Van Slyke method and corrected for the autolysis of the enzyme, has been expressed as the per cent of the total nitrogen of the original protein.

DISCUSSION

Peptic Hydrolysis—Two series of experiments were conducted to determine the type of linkage opened by pepsin. In the first series the concentrations of pepsin and lactalbumin were in a 1:9 ratio, and in the second, a 1:4.5 ratio. In the latter case the concentration of the pepsin was 8 mg. per cc. and the protein 36.3 mg. The concentration of the hydrochloric acid in these experiments was 0.175 N or 0.63 per cent, a quantity sufficient to maintain the pH between 1.6 and 2.2 during the entire experimental period. A control consisting of lactalbumin and acid and the usual enzyme control were carried out. In the first series, examinations were made immediately after mixing, at the end of 3, 21, and 23 days, whereas in the second, they were made at shorter intervals, as may be seen in Table I. The results of the first series are not given, since they are similar to those of the second.

The data show a 1:1 liberation of carboxyl and amino groups, which should result if the peptide linkage is broken. The data are concordant within the limits of the experimental error, except for the first three titrations by the Sørensen method. At the end of the titration in these instances the volume of the hydrolysate-formaldehyde-sodium hydroxide mixture was 23 cc. or less. In subsequent titrations, the dilution factor was avoided by increasing the amount of sodium hydroxide necessary to inhibit the digestion.

TABLE I

Digestion of Lactalbumin by Pepsin

The concentrations of lactalbumin and pepsin were 36.3 and 8 mg. per cc., respectively. The increases in titration are expressed in terms of cc. of 0.02 N solution for 5 cc. aliquots, *i.e.* for 181.5 mg. of lactalbumin. The increases in amino nitrogen (Van Slyke) have been calculated as cc. of 0.02 N solution per 5 cc. of aliquot. All results have been corrected for controls.

Digestion period	Titration			Amino N (Van Slyke)	
	Sørensen	Willstätter	Harris		
hrs.	*cc.*	*cc.*	*cc.*	*cc.*	*per cent total N*
1	4.8	6.6	7.1	6.7	7.0
$1\frac{1}{6}$				7.2	7.4
$1\frac{1}{3}$	5.7	7.4	7.6	7.6	8.0
4	7.7	9.8	9.7	9.9	10.4
8				10.4	10.9
24	13.8	14.8	13.4	14.2	14.9
168	18.6	18.8	17.4	17.2	18.6

The rate of hydrolysis during the early stages is also shown in Table I, at which time the digestion was very rapid. The cleavage effected during the 1st hour expressed as amino nitrogen was equivalent to 7.0 per cent of the total nitrogen, or more than one-third of that which occurred during 7 days, and in 4 hours it was 10.4 per cent, or more than one-half. At the end of 7 days, 18.6 per cent of the total nitrogen had been converted into detectable amino nitrogen.

After the rapid hydrolysis of the first few hours, the rate progressively decreased. The slow but definite cleavage of the later

stages was also demonstrated with eight other peptic hydrolysates, which were observed for periods of 28 to 76 days. In six of these with a pepsin-substrate ratio of 1:4.5, the increase in amino nitrogen by the 21st day was equivalent to 20.5 to 21.9 per cent of the total nitrogen. In four instances, where the experimental period was continued to the 52nd day, the total cleavage ranged between 22.5 and 23.7 per cent. The two most extensively cleaved digests, in which no detectable change was observed between the 66th and 76th days, had 24.1 and 24.7 per cent of the nitrogen converted into the amino form.

It was thought possible that the acid and not the enzyme was responsible for the slow, prolonged hydrolysis. To test this hypothesis, 10 cc. portions of the hydrolysates at various stages of digestion were removed, heated, and maintained at 80–85° for 5 minutes. They were then rapidly cooled to 30°, and the observations were then continued for 11 to 14 days. Heating these acid solutions did not produce any detectable change in the amino nitrogen. The various aliquots represented stages of digestion in which the increase in amino nitrogen prior to this heat inactivation of the pepsin ranged from 7.0 per cent of the total nitrogen to 24.3 per cent. In the lactalbumin-acid control for the experiments described above, the maximal change was equal to but 0.4 per cent of the total nitrogen in the course of 7 days. This value is within the experimental error that occurs when the lactalbumin is not in solution, as is true in this case. These observations indicate that the hydrochloric acid as used in these experiments caused no significant changes and could not have been responsible for the slow hydrolysis observed over long periods of time.

To ascertain the influence of the concentration of pepsin on the extent of hydrolysis, two kinds of experiments were planned. In one kind, all of the pepsin (three different concentrations being used in each series) was added to the substrate at the beginning of the experiment. In the other, more pepsin was added to hydrolysates which had been previously treated with pepsin and allowed to digest until no further increase in amino nitrogen could be detected.

Two series of the first type were carried out with similar results. In these the three concentrations of pepsin used were 8,

16, and 32 mg. per cc., so that the relationships of enzyme to lactalbumin were 1:4.5, 2:4.5, and 4:4.5, respectively. By the 17th day the percentages of hydrolysis in the first series were 19.3, 22.4, and 25.7 for the respective concentrations, and by the 28th day the percentages were 21.7, 23.3, and 26.0, respectively. The results for the second series are shown in Table II.

Six experiments of the second type were carried out. On the 29th day, 8 mg. of pepsin were added for each cc. of an aliquot of Hydrolysate VI. The cleavage effected in the following 29

TABLE II

Hydrolysis of Lactalbumin with Various Concentrations of Pepsin As Shown by Increases in Amino Nitrogen during Digestion

The concentration of lactalbumin was 36.3 mg. per cc.; of pepsin, 8, 16, and 32 mg. per cc. for Hydrolysates VI, VII, and VIII, respectively. All increases in amino nitrogen (Van Slyke) are expressed as per cent of total nitrogen.

Time	Hydrolysate VI	Hydrolysate VII	Hydrolysate VIII
hrs.	*per cent*	*per cent*	*per cent*
19	20.2	22.4	25.5
21	20.9	22.3	25.5
25	21.9	23.4	26.5
28	21.5	23.3	27.4
29	21.8		
31		23.2	
33	21.6		
34		23.4	26.8
37			28.0
42	22.8	25.0	28.1
53	23.7	24.6	29.0
60	23.9	24.9	28.3

day period was such that the total digestion was equivalent to 25.1 per cent of the total nitrogen, which was practically the same as that of Hydrolysate VII on the 42nd day, in which there was the same amount of pepsin (see Table II). Similarly, a further cleavage in Hydrolysate VII was caused by the addition of 16 mg. of pepsin per cc., which made the total quantity of enzyme used the same as in Hydrolysate VIII. In four cases, the amount of pepsin added to different hydrolysates was equal to 40 mg. for each cc. of the aliquots, making the total quantity

that had been used 48 mg. per cc., and an 8:6 ratio for the enzyme and substrate. The cleavages effected were equivalent to 7.9, 6.8, 7.7, and 6.7 per cent, or a total of 33.5, 32.2, 30.2, and 29.9 per cent, respectively. These data indicate quite clearly that the extent of digestion depends upon the concentration of the enzyme.

The cleavages may also be expressed in terms of the number of linkages broken by acid hydrolysis. The increase in amino nitrogen produced by heating the protein with 20 per cent hydrochloric acid for 32 hours was equivalent to 72.4 per cent of the total nitrogen. On the assumption that this represents complete hydrolysis (100 per cent), the hydrolyses effected by the various concentrations of pepsin would indicate a 29 to 39 per cent cleavage of the peptide linkages.

The extent of the hydrolysis of lactalbumin effected by pepsin is dependent upon the concentration of the enzyme. None of the observations reported here indicates that the maximal cleavage has been obtained as in the case of ovalbumin (5). For that protein where the amount of pepsin was about twice the quantity of substrate, no further digestion occurred after more enzyme was added. The cleavage was equivalent to 25.6 per cent of the total nitrogen or about one-third of the linkages opened by acid hydrolysis. In any experiment where such high concentrations of enzyme are used, the correction for the autolysis of the pepsin is large. Furthermore, this correction for the autolysis of pepsin in any concentration may not be accurate, as it is possible that the changes occurring in the enzyme control and in the digestion mixture may not be identical. The extent of cleavage observed for lactalbumin falls within the limits reported for various other proteins of high molecular weight, 20 to 40 per cent of the total nitrogen (5, 12–15).

The specific interpretation to be placed on the respective titrations depends upon the acceptance or rejection of the zwitter ion theory as applied to the products of peptic hydrolysis. According to the classical theory, the Sørensen and the Willstätter titrations measure the carboxyl groups, and the modified Harris the amino groups. But if the free amino and free carboxyl groups form an inner salt or a zwitter ion, the first two methods are essentially back titrations of the $—NH_3^+$, *i.e.* a conversion to an unionized amino group, and the third is a back titration of the

—COO^- group. If one considers that the products of peptic hydrolysis have a zwitter ion structure, then the 1:1 relationship between the Van Slyke data and those of the Sørensen and the Willstätter titrations cannot be interpreted as the liberation of one amino group for each carboxyl group. It may mean that no significant liberation of imino groups, which would be measured by these titrations but not by the Van Slyke method, has occurred, or that the inherent errors of the methods have masked the differences. Therefore, to relate the amino and carboxyl groups liberated, a comparison of the data obtained in the Van Slyke, Sørensen, and Willstätter methods should be made with that of the modified Harris. As the data obtained by all four methods employed showed a 1:1 relationship, the peptide groups only are opened, and neither ester linkages nor those involving the nitrogen of proline have been split. If these other linkages are hydrolyzed, the changes are so small that these methods are not sufficiently sensitive to detect them.

Action of Enzymes of Pancreas and Intestinal Mucosa on Peptic Hydrolysates of Lactalbumin—The hydrolysates used as substrates for this part of the study were those which had a pepsin-lactalbumin ratio of 1:4.5, as described in the first part. At the end of those experiments, the pepsin digests were heated with stirring at 80–85° for 5 minutes in a boiling water bath, cooled immediately, and stored in a refrigerator for 2 to 15 months. As two of the hydrolysates showed a slight increase in amino nitrogen during storage, Van Slyke determinations were made just prior to each subsequent experiment.

The procedure in these experiments was to adjust the hydrogen ion concentration of aliquots of the peptic hydrolysates and the pepsin controls to the required pH, then to add the enzyme preparation, make up to a definite volume, and immediately take samples for the titrations and the Van Slyke amino nitrogen determinations. In the experiments involving aminopolypeptidase and the extract of the intestinal mucosa, the pH of the mixtures was 7.5 and for all others the pH was 8.0.

Representative data of individual experiments and a summary of all the results are given in Table III.

Protaminase—The maximal hydrolysis was equivalent to 9.8 per cent of the total nitrogen. In some instances the digestion

TABLE III

Action of Enzymes of Pancreas and Intestinal Mucosa on Peptic Hydrolysates of Lactalbumin

In Columns 2 to 4 the values of representative experiments are given. In Columns 5 to 7 the total number of experiments in each series, the range of the hydrolyses effected by these enzymes of the pancreas or intestinal mucosa, and the range of the total cleavage are recorded. The same peptic hydrolysate (IX) was used in all experiments for which detailed results have been given except for the protaminase study in which Hydrolysate X was used. All increases in amino nitrogen (Van Slyke) are expressed as per cent of total nitrogen.

Enzymes (1)	Time (2)	Hydrolysis (3)	Total hydrolysis* (4)	No. of experiments (5)	Hydrolysis (6)	Total hydrolysis* (7)
	hrs.	*per cent*	*per cent*		*per cent*	*per cent*
Protaminase	0		21.3	8	4.3– 9.8	28.2–33.9
	24	7.2	28.5			
	48	8.6	29.9			
	68	9.8†	31.1			
Trypsinkinase	0		22.1	3	7.0– 8.6	29.2–33.4
	21	5.5	27.6			
	49	5.9	28.0			
	117	7.5	29.7			
Protaminase and trypsinkinase	0		22.1	3	10.0–14.1	35.0–36.4
	21	7.8	29.9			
	48	12.4	34.5			
	96	12.6	34.7			
	168	14.1†	36.2			
Protaminase, trypsinkinase, and carboxypolypeptidase	0		22.1	3	17.4–21.9	43.8–44.3
	18	16.0	38.1			
	90	16.2	38.3			
	120	21.9	44.0			
	144	21.9†	44.0			
Aminopolypeptidase	0		21.5	1	15.5–16.4	37.0–37.9
	12	2.2	23.7			
	39	6.9	28.4			
	96	13.6	35.1			
	168	14.5	36.0			
	192	14.5	36.0			
	216	15.5	37.0			
	240	16.4	37.9			
	264	15.5‡	37.0			

* Total hydrolysis is that due to pepsin plus that effected by the enzymes used in this series of experiments.

† Further addition of the enzyme preparations caused no further hydrolysis in 30 hours.

‡ No dipeptidase was detectable in the enzyme control when tested at this point.

TABLE III—*Concluded*

Enzymes (1)	Time (2)	Hydrolysis (3)	Total hydrolysis* (4)	No. of experiments (5)	Hydrolysis (6)	Total hydrolysis* (7)
	hrs.	*per cent*	*per cent*		*per cent*	*per cent*
Extract of intestinal mucosa	0		21.5	8	28.9–40.5	55.1–62.0
	24	27.3	48.8			
	48	31.8	53.3			
	72	33.9	55.4			
	96	36.2	57.7			
	120	38.6	60.1			
	168	40.3	61.8			
	192	40.5†	62.0			
Extract of pancreas	0		22.1	3	39.8–47.0	66.2–68.3
	144	45.6	67.7			
	192	45.6§	67.7			

§ Further addition of pancreatic extract caused no further hydrolysis in 30 hours. The addition of intestinal extract caused no further hydrolysis in 6 days.

was as low as 4.3 per cent, which may not have been maximal, because the preparation was not very active and the lack of material made it impossible to test for the completeness of the hydrolysis. Considering only the data from those experiments in which the completeness of digestion was established, the amino nitrogen liberated was equivalent to 9 or 10 per cent of the total nitrogen.

Trypsinkinase—With three different peptic hydrolysates, trypsinkinase effected similar degrees of hydrolysis, equivalent to 7.0, 7.9, and 8.6 per cent of the total nitrogen, respectively. These substrates varied in the extent of peptic digestion from 21.3 to 26.4 per cent of the total nitrogen. The latter value was for one of the hydrolysates in which the amino nitrogen had increased during storage so that the total digestion changed from 24.7 per cent of the total nitrogen to 26.4 per cent.

Carboxypolypeptidase—At the time that this experimental work was initiated, pure carboxypolypeptidase had not been isolated. Therefore, the action of this enzyme had to be determined by difference, with protaminase and trypsinkinase in one preparation, and these two enzymes plus carboxypolypeptidase in the other.

The cleavage effected by the combined action of protaminase

and trypsinkinase varied from 10.0 to 14.1 per cent of the total nitrogen, but the total cleavage in the three different hydrolysates was about the same. Therefore, the degree of hydrolysis produced by these enzymes is probably dependent upon the extent of the previous peptic digestion. The sum of the hydrolyses effected by the separate action of protaminase and trypsinkinase is approximately 17 per cent, whereas the simultaneous digestion by these two is 10 to 14 per cent. This would indicate that some of the same linkages are broken by each of these enzymes. In such a case, the calculated values would include the cleavage of some of the groups twice and would of necessity be greater than those observed for the simultaneous action of these two enzymes.

The extent of hydrolysis effected by the mixture of protaminase, trypsinkinase, and carboxypolypeptidase appears to depend upon the degree of the previous digestion, as was the case with the combined action of protaminase and carboxypolypeptidase. The range in values for the digestion produced by this mixture of 3 per cent enzymes was from 17.4 to 21.9 per cent of the total nitrogen, a difference of 4.5 per cent, whereas the difference between the highest and the lowest values for the total digestion (the above hydrolyses plus those previously due to pepsin) was only 0.5 per cent.

The hydrolyses due to carboxypolypeptidase, *i.e.* the difference between the action of the protaminase-trypsinkinase and the protaminase-trypsinkinase-carboxypolypeptidase mixtures, were 7.2, 7.8, and 8.0 per cent for Hydrolysates I, IX, and X, respectively (Table III). It cannot be assumed that this is the cleavage that this peptidase would have effected had the protaminase and the trypsinkinase not been present, since these two enzymes may furnish through their action additional substrate for the carboxypolypeptidase. Furthermore, carboxypolypeptidase might have split these linkages in the absence of the other two enzymes. However, this cleavage by carboxypolypeptidase is maximal under these experimental conditions.

Aminopolypeptidase—Difficulty was experienced in the preparation of aminopolypeptidase free of dipeptidase.[2] Two such

[2] The authors wish to express their appreciation to Dr. A. K. Balls for the colloidal iron oxide which was used in the separation of aminopolypeptidase and dipeptidase.

preparations were obtained, but in the first of these, dipeptidase became active during the course of the experiment. Therefore, only the results with the preparation which remained uncontaminated have been reported. In this instance, the cleavage was equivalent to 16 per cent of the total nitrogen, making a total hydrolysis of about 37.5 per cent. Complete digestion may be assumed, since no hydrolysis occurred during the last 48 hours of observation, and the enzyme control was active at the end of 264 hours toward the test substrate, leucylglycylglycine.

Dipeptidases of Intestinal Mucosa—As with carboxypolypeptidase, so here it was assumed that the difference in the digestion caused by the extract of the intestinal mucosa and aminopolypeptidase represents the action of dipeptidase or peptidases which have not been isolated. The absence of trypsinkinase was established, and since tests on many occasions had always shown that protaminase and carboxypolypeptidase were absent when there was no trypsinkinase present, they were not tested for in this instance.

The cleavage effected by this extract may be influenced by the extent of the previous peptic hydrolysis or by the age of the hydrolysate, as the two hydrolysates digested the least in this series of experiments were those which had been most extensively digested by pepsin and had been stored the longest following the heat treatment. In the hydrolysates in which the peptic digestion had been 21 or 22 per cent and storage 2 to 6 months, enzymes in this extract effected a maximal cleavage equivalent to approximately 40 per cent, the total hydrolysis being 61 to 62 per cent. With the two hydrolysates digested most extensively by pepsin and stored 10 to 12 months, the cleavage by the above extract was 28.9 to 29.6 per cent (maximal), giving a total digestion of 55.1 to 56.0 per cent. The significance of these observations is not recognized at present.

The difference between the digestion effected by this mixture of enzymes and by aminopolypeptidase is equivalent to approximately 24 per cent of the total nitrogen. These values cannot be assumed to represent the action of the dipeptidase alone, since the action of aminopolypeptidase and possibly other peptidases may have produced substrates cleavable by dipeptidase.

Combined Action of Enzymes in Pancreatic Extract—With the

three hydrolysates (I, IX, and X), the cleavage produced by the pancreatic extract was equivalent to 39.8, 45.6, and 47.0 per cent of the total nitrogen, respectively, making the total hydrolyses range from 66.2 to 68.3 per cent. These hydrolyses were maximal, as neither the addition of pancreatic extract nor that of the intestinal mucosa caused further digestion.

A comparison of the data obtained from the Willstätter titrations with those for the amino nitrogen as determined by the Van Slyke method shows in this series good agreement. For the majority of experiments, the values obtained by the two methods varied between 0.0 and 0.9 cc. of 0.02 N base per 100 mg. of lactalbumin, and in but nine cases out of thirty-eight were the differences more than 1.0 cc.

The cleavage of the peptic hydrolysates of lactalbumin by trypsinkinase, although not marked, is significant. This behavior of lactalbumin differs from that of ovalbumin, as with the latter no further digestion was effected by trypsinkinase following pepsin (5). Had the peptic hydrolysis of lactalbumin been maximal, as it was in the case of the other protein, trypsinkinase possibly would not have had any action.

One action of protaminase on the protamines is to split off the terminal arginine molecule on the carboxyl end of the chain. If this were the only action in the cases of lactalbumin, the maximal cleavage should have been equivalent to about 1.6 per cent of the total nitrogen, whereas the observed cleavage was 9.8 per cent. Similarly, the action of this enzyme on ovalbumin was greater than could be accounted for by arginine liberation. A study of the products of protaminase action should be made with special attention to the basic amino acids.

It is interesting that under the experimental conditions of this investigation the action of carboxypolypeptidase caused an increase in amino nitrogen equivalent to about 8 per cent of the total nitrogen, that of aminopolypeptidase 16 per cent, and that of dipeptidase 24 per cent; these are different from those cleavages observed with peptic hydrolysates of ovalbumin as substrates (5). The maximal hydrolyses effected by the extract of the pancreas were 6 to 11 per cent greater than those by the extract of the intestinal mucosa. The difference may represent the linkages that are broken by protaminase, trypsinkinase, or carboxypolypeptidase alone, or by their combined action.

The maximal enzymatic cleavage was equivalent to 94 per cent of the linkages broken by acid hydrolysis. This observation is in agreement with those for numerous proteins subjected to pepsin and unfractionated extracts of the pancreas and intestinal mucosa (16).

The interpretation of the comparison of the amino nitrogen by the Van Slyke method and the Willstätter titration as discussed above depends upon the zwitter ion theory. In view of these considerations, the agreement among the data obtained by these two different methods probably indicates that no significant number of imino groups has been liberated by these enzymes.

SUMMARY

1. During the peptic hydrolysis of lactalbumin, the peptide linkage is assumed to be the principal one broken since amino and carboxyl groups were liberated in equivalent amounts.

2. The rate of hydrolysis was rapid during the first 4 hours and then progressively decreased. A slow digestion, however, continued for 2 months or more, under the experimental conditions of the present study.

3. The hydrochloric acid (0.175 N) used in the maintenance of the pH of the peptic digests of lactalbumin played no significant rôle as a hydrolyzing agent under the experimental conditions employed.

4. The extent of hydrolysis was dependent upon the quantity of pepsin used. Under the experimental conditions of the present study, the number of the peptide linkages broken when the pepsin and lactalbumin were in a 1:4.5 ratio was about 29 per cent of those liberated by acid hydrolysis, or 21 to 22 per cent of the total nitrogen was converted into detectable amino nitrogen. The cleavage could be increased by the use of larger quantities of enzyme.

5. After digestion with pepsin, the hydrolysis effected by the various enzymes of the pancreas, as represented by the increase in amino nitrogen, was equivalent to approximately 10 per cent of the total nitrogen with protaminase, 7 to 9 per cent with trypsinkinase, 10 to 14 per cent with the combined action of these two, 17 to 22 per cent with the combined action of these two plus carboxypolypeptidase, and 40 to 47 per cent with the unfractionated extract.

6. In terms of increase in amino nitrogen, the hydrolysis effected

by aminopolypeptidase obtained from an extract of the intestinal mucosa was equivalent to 16 per cent of the total nitrogen, while with the unfractionated extract this was equivalent to 29 to 40 per cent of the total nitrogen.

7. The maximal enzymatic hydrolysis of the lactalbumin used in this investigation was obtained with pepsin plus the pancreatic extract, and was equivalent to 68 per cent of the total nitrogen or about 94 per cent of the linkages broken by acid hydrolysis.

8. To ascertain the type of linkage broken when the various enzymes in the extracts of the intestinal mucosa and the pancreas acted upon the peptic hydrolysates of lactalbumin, the Willstätter titration as well as the Van Slyke amino nitrogen determination was used. If the zwitter ion theory is accepted, the close agreement of the values obtained by the two methods may be interpreted as indicating that the linkages broken were those in which the nitrogen liberated was in the form of primarily amino groups and not imino groups.

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